Faculty of Resource Science and Technology

CHARACTERIZATION OF *Escherichia coli* FROM THE AQUACULTURAL ENVIRONMENT

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CHARACTERISATION OF *Escherichia coli* ISOLATED FROM THE AQUACULTURE ENVIRONMENT

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DECLARATION

I, Sheila Maria Andrew Stanley, hereby declare that this Final Year Project Report is my own work and effort that has not been submitted anywhere for any awards. Other sources and information obtained for this report are greatly acknowledged.

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Signature Date

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LIST OF ABBREVIATIONS

E. coli	*Escherichia coli*
kb	kilobase
µL	microlitre
µg	microgram
mL	millilitre
%	percentage
^{o}C	degrees Celcius
g	gram
DNA	deoxyribonucleic acid
MAR	multiple antibiotic resistance
PCR	polymerase chain reaction
dNTPs	deoxynucleotide triphosphates
RAPD	Rapid amplified polymorphic DNA
EMB	Eosin Methylene Blue
LB	Luria-Bertani
MHA	Mueller-Hinton agar

TSA	Tryptose Soy agar
^{o}	degrees
mm	millimetre
rpm	revolution per minute
$MgCl_2$	Magnesium chloride
mM	milli molar
µM	micro molar
dH_2O	distilled water
mins	minutes
bp	base pairs
AGE	agarose gel electrophoresis

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Characterisation of *Escherichia coli* isolated from the Aquaculture Environment

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ABSTRACT

Aquaculture environment is often exposed to contamination from the sewage and organic pollutants which are the main habitat for *E. coli*. Pathogenic *E. coli* can cause severe disease outbreaks through food consumption from aquaculture sources. Currently, antibiotics are used in the aquaculture industries for treatment of diseases among aquatic organisms, as a growth promoter, water treatment and pollution control. Nineteen *E. coli* isolates from the aquaculture environment were tested for their antibiotic resistance by disc diffusion method. The plasmid DNA extraction of the isolates was performed by alkaline lysis method. The genetic variations among the isolates were analysed by $(GTG)_5$ PCR. Based on antibiotic susceptibility test, 100% of the isolates were resistant towards erythromycin while the lowest level of resistance was against chloramphenicol and tetracycline (31.58%). Plasmid analysis revealed that 63.16% isolates contain plasmid DNA, whereas 36.84% are plasmidless. $(GTG)_5$ PCR analysis indicated that *E. coli* isolated were grouped into 2 main clusters and 6 minor clusters. This study proved that *E. coli* isolates from the aquaculture environment were highly diverse, as shown by their differences in antibiotic resistance patterns, plasmid profiles and $(GTG)_5$ PCR analysis. The multiple antibiotic resistance of the isolates indicates the potential hazard associated with antibiotic resistance bacteria from the aquaculture environment.

Keywords: *Escherichia coli*, antibiotics, plasmid profiles, $(GTG)_5$ PCR

ABSTRAK

Persekitaran akuakultur sering terdedah kepada pencemaran daripada sistem kumbahan dan bahan organik tercemar yang merupakan habitat utama untuk E. coli. Patogen E. coli boleh menyebabkan penyebaran wabak penyakit melalui pengambilan makanan dari sumber akuakultur. Kini, antibiotik telah luas digunakan dalam industri akuakultur untuk perawatan penyakit di kalangan organisma akuatik, sebagai penggalak pertumbuhan, perawatan air dan kawalan pencemaran. Sembilan belas E. coli dari kawasan akuakultur telah diuji untuk kepekaan terhadap antibiotik melalui kaedah penyerapan cakera. Pengekstrakan DNA plasmid telah dijalankan melalui kaedah pemecahan alkali. Kepelbagaian genetik antara E. coli telah dianalisa melalui $(GTG)_5$ PCR. Berdasarkan ujian kerintangan antibiotik, 100% daripada E. coli rintang terhadap eritromisin manakala tahap terendah adalah kerintangan terhadap kloramfenikol dan tetrasiklin (31.58%). Analisis plasmid mendedahkan 63.16% daripada E. coli mengandungi DNA plasmid manakala 36.84% tidak mengandungi plasmid. $(GTG)_5$ PCR menunjukkan E. coli telah dibahagikan kepada 2 kumpulan utama dan 6 kumpulan minor. Kajian ini membuktikan E. coli daripada persekitaran akuakultur mempunyai variasi genetik yang tinggi, sebagaimana kepelbagaian yang ditunjukkan dalam pola kerintangan antibiotik, profil plasmid dan analisis $(GTG)_5$ PCR. Kerintangan antibiotik berganda dari kalangan E. coli menunjukkan potensi yang tinggi untuk kehadiran bakteria yang rintang antibiotik dari persekitaran akuakultur.

***Kata kunci**: Escherichia coli, antibiotik, profil plasmid, $(GTG)_5$ PCR*

1.0 INTRODUCTION

The emergence of several antibiotic resistances among different bacteria species has been a great concern of public health in the world. Antibiotic resistance can be acquired from the widespread use of antibiotics among human which may lead to the transfer of resistance gene among the same or different bacteria strains (Wan *et al.*, 2003). As proposed by Sunde and Norstrom (2006), the distribution of antibiotic resistant bacteria between human and environment has been increased in food and water which may severely affect the national public health and development if the management of antibiotic usage is not being done properly.

Aquaculture industry is one of the main concerns as it can be an important medium for the emergence of antimicrobial-resistant *E. coli* and also other pathogenic bacteria. Some aquaculture products such as prawns, fish and oysters may contain their own normal microflora which can cause some negative effects towards the consumers. These products can be pathogenic when their microflora have possessed certain antibiotic resistance gene which can be directly transferred to human and it will possibly cause epidemic such as diarrhoea or food-borne disease (Sunde & Norstrom, 2006). Besides, food contamination is also one of the factors that enable the bacteria to exchange their genetic materials with one another (Kelly *et al.*, 2009).

Antimicrobial drugs have been widely used in the aquaculture environment. Despite of being used as a disease control, they are also required for the fisheries growth promoter in order to enhance meat production and nutritional value (Yoo *et al.*, 2003). However, the prolonged use of these drugs may lead to severe health problem due to the emergence of food-borne disease and thus, the production of agricultural products will be declining (Bischoff *et al.*, 2005).

E. coli is one of the common bacteria that can be isolated from the aquaculture environment. Enterotoxigenic *E. coli* (ETEC) is one of the major *E. coli* strains that affect the gastrointestinal tracts of the human by releasing its toxin which causes diarrhoea (Jayaratne *et al.*, 1987). As well as the other pathogenic strains of *E. coli*, this strain is also able to confer plasmid that codes for antibiotic resistance and enterotoxin production which can be transferred to the same or the other bacterial species through genetic recombination. The recombination of gene between two species has enabled the massive amount of bacterial population to acquire multiple resistances towards antibiotics.

The resistance gene may be transferred through conjugation, transduction or transformation (Dzidic & Bedekovic, 2003). The most common genetic transfer among bacteria is through conjugation which involves the cell-cell interaction. Conjugation between the same and different bacteria species is a type of horizontal gene transfer involving a communication between two bacterial cells which are the donor cell and recipient cells (Somkiat *et al.*, 2007). The recombination of the resistance gene from the donor and the antibiotic susceptible recipient will occur when the gene is integrated in the chromosomes or plasmid through conjugation process. Plasmid is a mobile element that

can increase the ability of bacteria to spread their resistance gene (Rijavec *et al.*, 2006). This has caused some changes in the recipient resistance phenotype when the new resistance gene from the donor integrates in their chromosomes. Therefore, the frequency of bacterial antimicrobial resistant will increase and it will lead to a serious global health problem.

The genetic diversity of *E. coli* isolates can be determined by several DNA fingerprinting methods. One of the DNA fingerprinting methods that can be used is the repetitive sequence-based PCR which is easy to perform based on repetitive sequenced primer complementary to the bacterial genome (Svec *et al.*, 2005).

The widespread of antibiotic resistance among bacteria in aquaculture environment has caused some major illness and problems in the pharmaceutical industries. This study will further investigate the risks associated with antibiotic resistance bacteria from the aquaculture environment.

The main objectives of this study were:

1) To determine the patterns of antibiotic resistance among the *E. coli* isolates.
2) To analyse plasmid DNA among the *E. coli* isolates.
3) To determine the genetic diversity among the *E. coli* isolates.

2.0 LITERATURE REVIEW

2.1 Aquaculture Environment

Aquaculture environment has been one of the most important medium in the agricultural industries for economic development (Sahu *et al.*, 2008). It provides the powerful source of protein from different species of aquatic organisms such as fish, prawns, oyster and also crabs. Cole *et al.* (2009) studied that approximately 40% of fish food is supplied by the aquaculture industries. However, these organisms are exposed to pathogens and other harmful microorganisms which lead to the massive loss of aquatic sources due to infections and diseases. Therefore, antimicrobial drugs have been extensively used in the aquaculture environment in order to control and prevent the spread of diseases.

The widespread use of antimicrobial drugs in the aquaculture environment has resulted in the serious concern towards the public medical and health sectors as the antimicrobial residues in the water and food sources may increase the population of multi-drug resistant microorganisms (Lu *et al.*, 2009).

2.2 *Escherichia coli*

Escherichia coli (abbreviated as *E. coli*) is a type of gram negative bacilli which uses its own flagella to move. It exists as a normal microflora in the digestive tract of human and the other warm-blooded organisms. Most of *E. coli* strains are considered as harmless but there are certain types of *E. coli* that are pathogenic and may cause severe effects towards the environment. *E. coli* is classified into six classes which are enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), Shiga-toxin producing (STEC), enteroaggregative (EAEC) and diffuse adhering (DAEC) (Blanco *et al.*, 2006). All of these six classes of *E. coli* are categorised as pathogenic as they are able to infect their host and exhibit some antibiotic resistance through genetic transfer inside the host.

Most of *E. coli* strains carry resistance factors (R factors) in their plasmid which encodes for antibiotic resistance (Wan *et al.*, 2003). These R factors are transferable among the same *E. coli* species or to the other kinds of bacteria in order to establish multiple antibiotic resistances through genetic transfer. Antibiotic resistance develop when the resistant strains infected their host and transfer their plasmid into the host's DNA. These strains may also interact with the normal microflora in the gastrointestinal tract. This will cause the normal flora in the human body to establish certain resistance towards antibiotics which increase their pathogenicity towards the host. Pathogenic *E. coli* strains can also encode for various virulence determinants on their individual chromosomes and plasmids (Ambrozic *et al.*, 1998). Therefore, their resistance towards multiple drugs will increase

and cause failure to treat the disease which will lead to a major outbreak (Dzidic & Bedekovic, 2003).

E. coli strains are reported to have increase in their resistance which cause many disease outbreaks in countries such as clinical diarrhoea due to their presence in the contaminated food and water (Wan *et al.*, 2003). Previous study investigated that *E. coli* have a great activity against tetracycline, sulphonamide and amynoglycoside in farm animals (Yang *et al.*, 2004). Hence, this has cause a concern towards the treatment for infected farm animals, especially in the aquaculture environment which provides the highest protein source across the countries. Consequently, the emergence of multiple antibiotics resistance among the *E. coli* isolates will greatly influence the economy and farm industries.

2.3 Antibiotic Resistance among Bacteria

2.3.1 Antibiotic Resistance

Antibiotic resistance is an ability of bacteria species to resist certain drugs in specific concentration which will indirectly increase the pathogenicity of the bacteria (Varaldo, 2002). The increase of pathogenicity of these species may be due to the transferable resistance genes that occur progressively within the environment. As reported by Varaldo (2002), the antimicrobial resistance among bacteria which is related particularly towards

the pathogenicity has been emerged over the last few years. Antibiotic resistance gene can be transferred to a wide varieties of bacteria species, regardless of the gram negative or gram positive strains. These genes can be transferred directly from the gram negative donor to the gram positive recipient or to the same group of bacteria strains.

The resistance of bacteria towards certain antibiotics can be tested by antimicrobial susceptibility test through disc diffusion method (Wan *et al.*, 2003). Bacterial strains will be cultured in an agar media, such as commonly used Mueller-Hinton agar to investigate the susceptibility of certain bacterial strains against antimicrobial drugs. The zone diameter interpretive standards obtained from the test are the standard measurement applied to determine the lowest concentration of antibiotics used for bacterial growth inhibition against the drugs (Wikler *et al.*, 2006).

2.3.2 Types of Antibiotics

Antibiotic is a type of antimicrobial drugs that is able to kill or inhibit the bacterial growth. Antibiotics are chemically derived from microorganisms which enable them to eliminate pathogens that surround them. Antibiotics can be bactericidal or bacteriostatic. Bactericidal antibiotics act by killing bacteria, whereas bacteriostatic antibiotic acts by inhibiting the bacterial growth (Pankey & Sabath, 2004). Every antibiotic has their own spectrum range, which indicates their antimicrobial activity towards certain bacteria. Antibiotics with a broader spectrum range will be able to inhibit a large population of gram positive and gram

negative bacteria. Meanwhile, a narrow spectrum range of antibiotics are only able to eliminate a few types of bacteria (Perlman, 1977).

Various groups of antibiotics have been identified according to their spectrum range and their effectiveness towards certain infections in the medical treatments. The main groups of antibiotics are macrolides, aminoglycosides, penicillins, tetracyclines, fluoroquinolones and cephalosporins. Macrolides are a type of erythromycin antibiotics which acts by binding upon the the ribosomes of the bacteria to inhibit protein productions (Leclercq, 2002). Meanwhile, aminoglycosides are broad spectrum antibiotics which act towards gram negative bacteria by inhibiting their protein synthesis through binding to 30S ribosomes (Mingeot-Leclercq *et al.*, 1999). Penicillins have the ability to kill susceptible bacteria by preventing the final stage of bacterial cell wall synthesis which causes fatality towards the bacteria. Besides that, tetracyclines are also widely used as the anti-inflammatory broad spectrum drugs which are able to inhibit the tumour progression (Amin *et al.*, 1996). Other broad spectrum antibiotics that are extensively used in medical term are fluoroquinolones which kill bacteria by disrupting the bacterial DNA synthesis and cephalosporins which inhibit the synthesis of bacterial cell walls.

2.4 Bacterial Plasmid DNA

In spite of bacterial DNA, plasmid DNA also exists in some bacterial strains and it may contain information about the diversity of bacterial genetics. According to Jan *et al.* (2009), plasmid is one of the main medium for the transfer of antibiotic resistance among bacteria. As well as bacterial DNA, plasmid has its own replication system which encodes for the plasmid behaviour and characteristics for bacterial pathogenicity because one of them might contain several plasmids that code for different antimicrobial resistance.

Plasmid DNA is also a mobile element that enables the bacteria to spread its resistance potential to the other bacterial strains through genetic recombination (Rijavec *et al.*, 2006). This mobile element can encodes for both antibiotic resistance and virulence factor that can cause the replication of more virulent antibiotic resistant strains. Rijavec *et al.* (2006) also reported that the most prevalent strains of *E. coli* are resistance towards tetracycline and chloramphenicol. These antibiotic resistances are found to be associated with the behaviour of conjugal plasmid of *Enterobacteriaceae* which primarily promotes the transferable plasmids into other strains in the environment.

Currently, plasmids are used as a vector for genetic engineering because of its capability of transferring a specific gene from one cell to another. Besides, plasmids have different sizes that enable differentiation between two bacterial strains which is crucial for plasmid profiling.

2.4.1 Alkaline Lysis

Alkaline lysis is one of a method used in the plasmid DNA extraction. It was first proposed by Birnboim and Doly (1979). This step involves the differential denaturation and reannealing of plasmid DNA, as compared to the chromosomal DNA (Ehrt & Schnappinger, 2003). Alkaline lysis consists of three main steps, which are resuspension, lysis and neutralisation. As stated by Kado and Liu (1981), chromosomal DNA and plasmid DNA can be obtained directly through the disruption of cell, or as well known as lysis. Lysis can be done by the mean of lysozyme action and detergent. Therefore, a series of centrifugations will be performed to assist in a better plasmid DNA extraction.

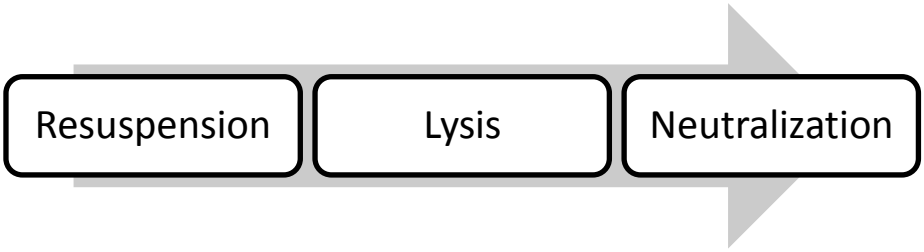

Figure 1: Overall steps involved in alkaline lysis for plasmid DNA extraction

2.4.2 Plasmid DNA Profiling

The study of prevalence of bacterial strains towards antibiotic resistances can be further investigated through plasmid profiling. Antibiotic resistance gene among bacteria can be carried within the plasmid DNA which contributes to the increased populations of bacteria that confers multiple drug resistance (Rijavec *et al.*, 2006). Plasmid identification and characterisation act as the main role for epidemiology investigation as this mobile element has created a great genetic diversity, primarily towards the bacterial expressions against antibiotics and pathogenicity (Jan *et al.*, 2009).

Plasmid profiling also enables the measurement of antibiotic resistance and virulence factors of some bacteria as plasmid may contain the R factor carrying the resistance gene which can be distributed in different sizes as well in the chromosomal DNA (Alam *et al.*, 2010). Therefore, different sizes of plasmid DNA in every bacterial strain will enable the identification and characterisation of plasmids for profiling purpose.

2.5 Genetic Recombination as the Main Medium for Antibiotic Resistance Transfer

2.5.1 Horizontal Gene Transfer

Horizontal gene transfer of two bacteria of the same and different species involves the transfer of genetic material forming a transconjugant bacterial species that encounter a new phenotype. As stated by Somkiat *et al.* (2007), conjugation between different bacteria strains involved a horizontal gene transfer process by which the donor possessing conjugative plasmid will transfer its resistance to the plasmid-free recipients. The transfer of antibiotic resistance between two bacteria enables the donor cell to transfer certain antimicrobial resistant gene to the antibiotic susceptible recipient while the donor interacts closely with the recipient. This phenomenon has cause a major increase in antimicrobial resistance in some normal flora in the environment which makes them become pathogenic towards human and the other organisms.

Horizontal gene transfer can be divided into three mechanisms which are conjugation, transduction and transformation (Hassan & Amin, 2010). These mechanisms have been reported as one of the means that contributed into the evolutionary of recombinant bacteria which is useful for the agricultural and bioremediation purpose. However, bacteria pathogens also may emerge due to some mutation during the recombination and it will attract some major attention around the world. Furthermore,

bacteria that have acquired certain antibiotic resistance will be able to spread their pathogenicity to the other bacteria and may create pandemic.

2.5.1.1 Conjugation

Conjugation is a type of horizontal gene transfer that involves the transfer of conjugative plasmid which carries certain resistance gene through cell-cell interaction (Kelly *et al.*, 2009). Certain genes that encode for antibiotic resistance are often linked to mobile genetic elements such as transposon and plasmid which primarily occur in the bacterial chromosomes (Sunde & Norstrom, 2006). During conjugation, the resistance gene will be transferred by forming a sex pilus which is made by the donor cell (NCBE, 2009). Sex pilus appears in a form of tube which will provide a pathway for the plasmid to be transferred from the donor strain to the recipient strain. Plasmid DNA from the donor will pass through the sex pilus as a single stranded DNA and when it is arrived upon the recipient cell, the single-stranded DNA will either replicate to form the previous plasmid circular shape or integrate into the recipient's chromosome (NCBE, 2009).